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A DOTA–peptide conjugate by copper-free click chemistry

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ABSTRACT

Attachment of DOTA to a novel monofluoro-cyclooctyne facilitates bioconjugation to an azide-modified peptide via Cu-free click chemistry. The resulting conjugate was radiolabeled with ^{111}In to afford a potential targeted molecular imaging agent with high specific activity and an excellent radiochemical purity.

Numerous synthetic approaches have been applied to add radionuclide chelators, such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), to peptides and other molecular targeting vectors to produce bifunctional molecular targeting agents. Addition of the chelator is required to maintain a stable radiometal-peptide coupling for applications in targeted molecular imaging and therapy.[1–5] Reactive groups present in DOTA derivatives that have proven useful for these applications include active esters, *para*-isothiocyanato derivatives, and terminal amine functional groups. One particularly advantageous approach for chemical conjugations is the use of so-called 'click' chemistry, which has received remarkable attention over the past several years for use in numerous applications in bioconjugate chemistry.[6] The most widely-used click reaction is based on [3+2] cycloaddition reactions between azides and alkynes to form triazoles as first described by Huisgen.[7–10] Copper(I) catalyzed variants of this cycloaddition have been subsequently developed that lead to triazole products in high yields under mild conditions.[8–11] For example, El-Sagheer and Brown highlight the advantages of the reaction for preparation of a modified DNA for numerous applications.[7] Significantly, very few publications have reported the use of copper catalyzed click reactions for the coupling of metal chelators (e.g., DOTA) to bioactive molecules such as peptides and aptamers.[12] This is primarily due to the high affinity of the Cu catalyst for the macrocyclic chelator moiety, which can interfere with subsequent radiolabeling reactions, thereby reducing the achievable specific radioactivity of the desired bioconjugate. Recently, however, Lebedev et al., described the facile preparation of DOTA–peptide conjugates by Cu(I) catalyzed azide–alkyne click chemistry.[13] Copper was removed from the bioconjugated chelators by precipitation with sodium sulfide Na_2S and this work represents a promising route for preparation of bioactive radiolabeled agents. We have also attempted to utilize Cu(I)-catalyzed click reactions for the preparation of various DOTA-biomolecule conjugates using a similar approach described by Knor et al.[12] While the cycloaddition reactions themselves were successful, the ability to remove the copper catalyst from our reaction mixtures proved troublesome. This was particularly true in the case of the preparation of DOTA–RNA aptamer constructs in which the conditions for the copper sulfide precipitation were corrosive to our nucleic acids and residual Cu coupling was observed in ESI mass spectral analysis of the compounds following application of the precipitation. Further, we were convinced that the presence of Cu would inhibit radiolabeling of DOTA-modified bioconjugates, reducing the achievable specific radioactivity for imaging applications. These experiences led us to examine the feasibility of employing Cu-free click reactions.[14] Specifically, it has been demonstrated that ring strain inherent in cyclooctynes promotes spontaneous cycloaddition with organic azides under mild conditions and in the absence of copper catalysts.[15–18] We thus considered construction of a

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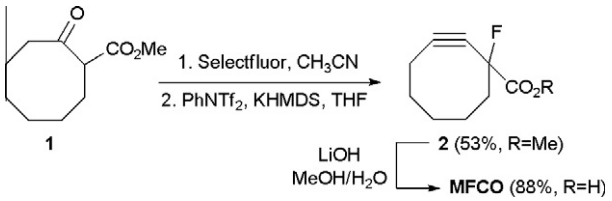
DOTA derivative functionalized with a modified cyclooctyne moiety that could then be attached to molecular targeting vectors via copper-free click chemistry. Numerous apparently suitable cyclooctyne ligands for bioconjugate applications have been advanced. Notably however, as pointed out by Jewett and Bertozzi, preparation of each of these functional moieties presents problematic steps.[14] Our investigations along these lines resulted in development of an efficient, tractable, high-yielding three-step synthesis of a versatile monoflouro-substituted cyclooctyne (MFCO) that potentially could be used to facilitate a variety of bioconjugation processes (Scheme 1).[19] We provided proof of concept copper-free click addition of a chelator to a relevant small molecule (biotin) that illustrated the usefulness of the MFCO as a lynchpin for conjugation of a reporter moiety to a small molecule for possible applications in molecular imaging by radiolabeling with positron emitting radionuclide gallium-68 (^{68}Ga).

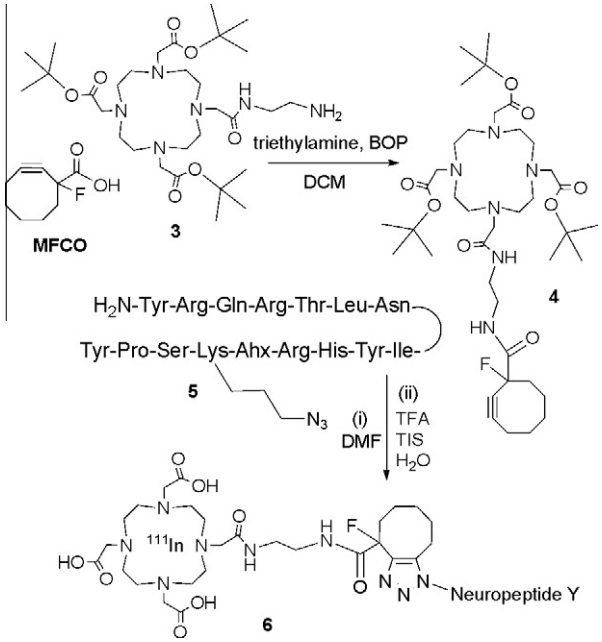
In this Letter, we demonstrate the usefulness of MFCO for preparing chelator-modified peptide-based molecular targeting vectors by copper-free click chemistry. We employ an easily prepared peptide-derivative (DOTA-MFCO) for copper-free click chemical addition at an internal azide-modified Lys residue of a neuropeptide Y (NPY) analog, which is under examination for use in molecular imaging of childhood neuroblastoma.[20] This approach provides a straightforward route to a targeted molecular imaging agent that exhibits high specific activity and excellent radiochemical purity when reacted with gamma-ray emitting radionuclide indium-111 (^{111}In), which is commonly employed for single-photon emission computed tomography (SPECT). Notably, this study also highlights the potentially general utility of DOTA-MFCO reagents such as **4** for assembly of bioconjugates relevant to targeted molecular imaging.

The synthesis of MFCO was achieved using a route that we described previously.[19] Briefly, methyl-2-oxo-cyclooctane carboxylate **1** was fluorinated with Selectfluor (Scheme 1). The resulting monofluoro-cyclooctanone was then converted to the cyclooctyne **2** in one pot via elimination of an in situ-generated vinyl triflate. Finally, simple saponification of the methyl ester afforded MFCO. Incorporation of the fluoro substituent serves two purposes. First, fluorination of **1** produces a quaternary center adjacent to both carbonyl groups, thereby facilitating chemo- and regioselective enolization of the ketone in the next step. Second, the presence of electron-withdrawing fluorine atoms adjacent to the strained alkyne is known to further increase the rate of dipolar cycloaddition with azides.[16–19]

With MFCO in hand, a DOTA-MFCO–NPY conjugate was prepared by first coupling an amine-modified DOTA **3** to MFCO, then conjugating the DOTA-MFCO **4** to the azide on Lys^4 of the NPY peptide **5** (Scheme 2). Briefly, MFCO (1 equiv) was dissolved in dichloromethane (DCM), and triethylamine (1 equiv) and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (BOP; 1 equiv) were added. To this mixture was added an amine modified and *tert*-butyl protected DOTA derivative **3** (1.1 equiv), and more triethylamine (1.1 equiv). The reaction mixture was mixed gently at 25 °C for 24 h, at which time the solvent was removed by rotary evaporation. The product (DOTA-MFCO **4**) was characterized by mass spectrometry, and used in the following steps without further purification (Fig. S1).



Scheme 1. Synthesis of MFCO.



Scheme 2. Synthesis of DOTA-MFCO (**4**); and DOTA-MFCO–NPY bioconjugate (**6**, 13% overall yield) and radiolabeling with ^{111}In.

Neuropeptide Y derivative **5** [Lys(N$_3$)^4,Ahx$^{1-24}$]NPY[21] was synthesized on a 0.10 mmol scale on Rink resin using standard Fmoc procedures (see Supplementary data). For peptide conjugation, the lysine at position 4 (Lys^4) was replaced with an azide derivative for reaction with **4** by 'click' chemistry. DOTA-MFCO was conjugated to the azide-modified side chain of Lys^4 using an approximate 10:1 molar ratio of DOTA-MFCO–NPY in DMF for 24 h at 25 °C. The DOTA-MFCO–NPY bioconjugate **6** was simultaneously cleaved from the resin and deprotected by 95:2.5:2.5 trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/water (v/v/v) and then precipitated with ice cold ether and purified by semipreparative high performance liquid chromatography (HPLC, Fig. S2).

Radiolabeling experiments were carried out using the DOTA-MFCO–NPY bioconjugates and single-photon emitting radionuclide ^{111}In. Serial dilutions of the DOTA-MFCO–NPY conjugate **6** were prepared from 12 to 1.5 nmol of DOTA–peptide in 200 μL of sodium acetate/acetic acid buffer (pH 4.5). To these solutions was added ^{111}In aliquots of approximately 135 MBq each, dissolved in identical buffer. The reaction mixtures were incubated at 99 °C for 30 min with continuous mild agitation. These samples were then cooled and analyzed sequentially without further purification by radioHPLC (Fig. 1). Radiolabeling produced excellent results at each concentration of DOTA–peptide conjugate leading to specific activity as high as 88 MBq nmol^{-1} (836 mCi mg^{-1}) and radiochemical purity in excess of 98% without additional purification (Fig. S3). This value of specific activity compares favorably with the maximum achievable specific activities reported for radiolabeling of peptide conjugates with ^{64}Cu that were prepared using copper-catalyzed conjugation strategies.[13] These authors report a maximum achievable specific activity of 250 mCi mg^{-1} for a chelator modified peptide, with an HPLC purification required to remove free ^{64}Cu. Nonetheless, the procedure described by Lebedev et al. represents an attractive alternative and a direct comparison of the approaches is a subject of future research in our laboratory.

We found that at peptide concentrations less than 1.5 nmol of the DOTA-conjugate (given ^{111}In of ~130 MBq) that a purification step to achieve our target of >98% radiochemical purity would be required to remove excess 'free' ^{111}In within the constraints of the 30 min radiolabeling step at 99 °C and pH 4.5 (data not shown).

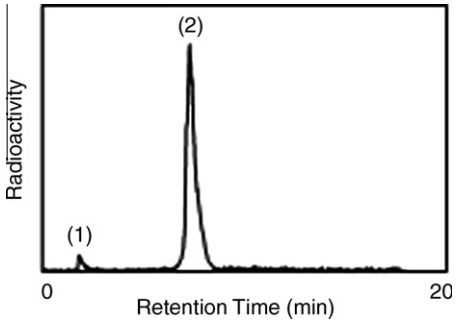


Figure 1. Radio-HPLC analysis of ^{111}In-labeled DOTA-MFCO–NPY (1.5 nmol) conjugate; (1) retention time of 'free' (unlabeled) ^{111}In; (2) retention time of ^{111}In-labeled DOTA-MFCO–NPY; >98% radiochemical purity; and specific activity of 88 MBq nmol^{-1}.

Our radiolabeling conditions are about 0.5 pH units above published accounts of optimum conditions for radiolabeling of peptides with ^{111}In, but consistent with our experience in achieving high specific activities.[21] Avoiding the post-labeling purification step is advantageous for molecular imaging applications. Documentation of stable metal impurities provided by the ^{111}In manufacturer include major impurity stable aluminum (Al^{3+}) and to a lesser extent iron, copper, lead, and nickel. Experiments were conducted at approximately one half life post production of ^{111}In, thus allowing for significant ingrowth of stable Cd^{2+}, which is also known to inhibit radiolabeling of DOTA–peptide conjugates.[21]

In summary, through this proof of concept study we have successfully conjugated a novel DOTA derivative to a neuropeptide Y analog using copper-free click chemistry. Subsequent radiolabeling of this assembly with ^{111}In was achieved in high radiochemical purity, and the radiolabeled material displayed specific activity approaching 90 MBq nmol^{-1}. While the peptide targeting agent employed in this study is under investigation for molecular imaging of childhood neuroblastoma, the approach described above should be compatible with the preparation of a wide range of peptide–DOTA bioconjugates. Indeed, it is envisioned that key intermediate **4** may serve as the prototype for a family of versatile reagents for the introduction of macrocyclic chelators to a variety of biomolecules via Cu-free click chemistry. The ready availability of cyclooctyne MFCO using a straightforward three-step synthesis should facilitate these efforts for this general approach. Optimization of the reaction conditions for the copper-free click chemical addition using the DOTA-MFCO and other MFCO functionalized chelators for preparing peptide conjugates suitable for molecular imaging is a subject of continuing research in our laboratories.

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Supplementary data

Supplementary data (details of synthesis, purifications, radiolabeling, radioanalytical HPLC and mass spectrometry results) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.111.

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